

The University of Chicago

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NUM, CHROMIUM, AND THE RARE
EARTHS IN THE SUCCINIC DEHY-
DROGENASE-CYTOCHROME SYSTEM**

**II. THE INHIBITION OF SUCCINIC DEHY-
DROGENASE BY COPPER**

**A PART OF A DISSERTATION SUBMITTED TO
THE FACULTY OF THE DIVISION OF THE
PHYSICAL SCIENCES IN CANDIDACY FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY**

DEPARTMENT OF CHEMISTRY

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By

BERNARD L. HORECKER

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THE PROMOTING EFFECT OF ALUMINUM, CHROMIUM, AND THE RARE EARTHS IN THE SUCCINIC DEHYDROGENASE-CYTOCHROME SYSTEM

By B. L. HORECKER

In the course of an investigation on the succinic dehydrogenase-cytochrome enzyme system, γ -aluminum hydroxide was used in an attempt to isolate certain of its components. The inconsistency of the results and the increased total activity obtained by this procedure suggested that some important factor was introduced by this treatment. Studies with such a factor in view showed clearly that aluminum ion addition in very small concentrations accelerated the aerobic oxidation of succinate markedly. Subsequent studies showed that aluminum is not unique in this accelerating property, but that it may be substituted by trivalent chromium and certain rare earths but not by ferric ion or by divalent ions. However, spectroscopic examination showed that of these metals only aluminum was present in the enzyme preparations in amounts sufficient to be of any significance. The following is a report of these findings.

EXPERIMENTAL

After it was determined qualitatively that the addition of aluminum ion, even in as small a concentration as that afforded by a saturated aluminum hydroxide solution, accelerated the rate of oxygen uptake in a succinic dehydrogenase-cytochrome system, a systematic series of quantitative studies was made.

The experiments were carried out at 38° in Warburg Erlenmeyer flasks. The substrate, 0.3 cc. of 0.2 M succinic acid previously neutralized with NaOH, was added to the side arms. 0.1 cc. of cytochrome C and 1.0 cc. of 0.1 M phosphate buffer (pH 7.15)

were added to each flask. Pure cytochrome C was prepared according to Keilin and Hartree (1) and was found by spectrophotometric analysis to contain 9.1×10^{-5} mm per cc. The total volume of reactants was always 3.3 cc. To prevent possible denaturation of the enzyme, the unbuffered aluminum chloride solution was always added last. The enzyme used was prepared by extracting washed minced beef heart muscle with alkaline phosphate according to a modification of the method of Stotz and Hastings (2) in which the alternate washings with warm

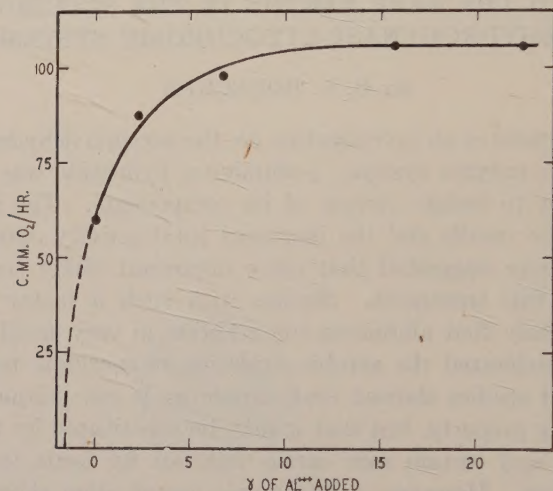


FIG. 1. The effect of increasing aluminum concentration on the rate of oxygen uptake of the succinic dehydrogenase-cytochrome C system. $T = 38^\circ$, pH 7.15, succinate 0.06 mm total, cytochrome C 9.1×10^{-5} mm total, 0.1 cc. of enzyme.

water were omitted. In most cases this crude preparation was twice precipitated with 0.2 M acetate buffer of pH 4.5 and re-suspended in phosphate buffer of pH 7.15. Unless otherwise specified, this will be the enzyme preparation referred to in all the following experiments.

With increasing concentrations of aluminum, the acceleration of succinate oxidation increased and approached a maximum value. A typical determination of this kind is shown in Fig. 1. By extrapolating the curve in Fig. 1 to zero activity (dotted line) it should be possible to estimate the aluminum concentration

in the original enzyme preparation, assuming the aluminum was necessary to the system. This estimated value (1.5 micrograms per 0.1 cc.) was then compared with the spectrographically determined value for aluminum in an enzyme preparation and was found to be the same, within 50 per cent error. Chromium was found present in very much smaller concentrations, less than 0.10 microgram per 0.1 cc. We wish to express our appreciation to Professor W. C. Pierce for his cooperation in making these analyses.

TABLE I
Activation by Various Metal Ions

$T = 38^\circ$, succinate 0.06 mm total, pH 7.15, cytochrome C 9.1×10^{-6} mm total, enzyme 0.1 cc.

Metal added	Amount of metal ion (total)	Velocity	Increase
		<i>c.mm. O₂ per hr.</i>	<i>per cent</i>
None.....	0.0	69.9	
AlCl ₃	83.0 mm $\times 10^{-5}$	124.2	77.7
CrCl ₃	40.0 " $\times 10^{-5}$	183.0	162.0
NdCl ₃ *.....	100 micrograms salt	123.9	77.4
La(NO ₃) ₃ *.....	100 " "	149.4	114.0
NaSm(SO ₄) ₂ *.....	100 " "	145.4	115.0
None.....	0.0	62.5	
Mg (C ₂ H ₃ O ₂) ₂	25.0 mm $\times 10^{-5}$	67.4	
Mn(NO ₃) ₂	20.0 " $\times 10^{-5}$	39.1	
ZnSO ₄	20.0 " $\times 10^{-5}$	0.0	
None.....	0.0	113.5	
FeCl ₃	40.0 mm $\times 10^{-5}$	99.0	

* The rare earth salts had a purity of 90 to 99 per cent. The impurities consisted of salts of other members of the rare earth group.

To determine whether aluminum was unique in possessing this accelerating property, the effect of the addition of various other salts was determined. As shown in Table I only the trivalent ions were effective. The exception in the case of ferric ion might be accounted for by the extreme insolubility of the hydroxide (pH of the reaction mixture = 7.15). Chromium was found to be more effective than aluminum, while zinc inhibited completely. A study with varying concentrations of chromium gave results

similar to those obtained with aluminum (Fig. 1) except that chromium was in general about twice as active, mole for mole, and also gave a maximum which was twice that for aluminum. A very similar effect was observed by Lohmann and Schuster (3) and Ochoa and Peters (4) on the accelerating action of magnesium and manganese in the cocarboxylase system. Attempts to prepare an inactive preparation of succinic dehydrogenase which could be reactivated by adding aluminum were unsuccessful because of the inactivation of the enzyme by long dialysis.

Function of Aluminum

To determine whether the aluminum affected the cytochrome oxidase portion of the system or the succinic dehydrogenase, experiments were performed in such a way as to allow each of these parts to operate independently.

The physiological aerobic oxidation of succinate is now considered to be (a) the reduction of cytochrome C by succinate in the presence of succinic dehydrogenase, and (b) the oxidation of the reduced cytochrome by oxygen in the presence of cytochrome oxidase. Although Ball (5) has shown that cytochrome B under equilibrium conditions is 75 per cent reduced by succinic acid, this mechanism is probably of secondary importance to the cytochrome C system.

In a previous paper (6) it was shown that small concentrations of cyanide inhibit the aerobic oxidation of *p*-phenylenediamine to the extent of 85 to 90 per cent and that a very large concentration of cyanide is necessary for the complete inhibition. On the basis of Keilin's previous work (7) that the autoxidation of cytochrome B is not inhibited by small quantities of cyanide, it was assumed that the non-cyanide-sensitive portion of the system acting on *p*-phenylenediamine was very probably cytochrome B. In this investigation we have found that the aerobic oxidation of succinate is likewise inhibited only to the extent of 90 per cent by small concentrations of cyanide. In this respect the aerobic oxidation of *p*-phenylenediamine and that of succinate are very similar. Aluminum ion addition did not accelerate the "residual" aerobic oxidation of succinate after the addition of cyanide. We may therefore conclude that aluminum ion very probably does not affect the aerobic oxidation of succinate through cytochrome B.

Both *p*-phenylenediamine and hydroquinone are capable of rapidly reducing cytochrome C without the intervention of a specific dehydrogenase and, as substrates (with excess cytochrome), can be used as a measure of cytochrome oxidase activity (6). With systems in which these substrates are used aluminum gave no acceleration. We can therefore conclude that it does not affect the so defined cytochrome C oxidase portion of the system.

By inhibiting the action of cytochrome oxidase with cyanide, the succinic dehydrogenase activity can be tested with methylene blue as a hydrogen transporter. In this case too the addition of aluminum gave no acceleration, and we are forced to conclude that the aluminum does not affect this portion of the system as determined by this test.

When aluminum was added in large concentrations to the reaction mixture (lacking enzyme), a precipitate, presumably of aluminum hydroxide, was formed. With enzyme present this precipitate was much more voluminous, indicating adsorption or coprecipitation of some constituent. In the latter case the supernatant liquid obtained by low speed centrifugation was less active than the uncentrifuged suspension. Furthermore it was found that the precipitate itself was active. While no precipitate was observed with small aluminum ion addition, the natural turbidity due to the enzyme made the observation of such a precipitate very difficult.

We are inclined to believe that the explanation of the increased activity is in some way bound up with the precipitate formation and this action can only be satisfactorily interpreted after the succinic dehydrogenase-cytochrome system is better understood.

SUMMARY

1. The addition of aluminum ion to a succinic dehydrogenase-cytochrome oxidase system accelerates the rate of oxygen uptake. A limiting value of the acceleration is reached with very small amounts of aluminum.

2. Spectrographic analysis of the enzyme preparations indicated that the amount of aluminum found in these preparations was sufficient to account for the activity, assuming that aluminum is a necessary constituent.

3. Chromium and the rare earth salts produced the same effect but were found to be practically absent in a tissue extract.

4. The function of aluminum in succinate oxidation is discussed.

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Inhibition of Succinic Dehydrogenase by copper

BY

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With 1 figure.

(29.VIII.39.).

QUASTEL and WOOLRIDGE¹⁾ found that copper sulfate inhibited completely the succinic dehydrogenase activity of *B. coli*. HOPKINS, MORGAN and LUTWAK-MANN²⁾ demonstrated that small amounts of copper could completely inhibit the activity of succinic dehydrogenase in minced rabbit muscle. On the basis of other evidence presented, the latter investigators conclude that the activity of succinic dehydrogenase is associated with the SH groups in the protein. The inhibitory effect of copper is thus explained as the result of its ability to bind SH groups.

In a study of the aerobic activity of succinic dehydrogenase preparations from beef heart, it was observed that a marked increase in the rate of oxygen uptake of the succinic dehydrogenase-cytochrome system could be produced by the addition of low concentrations of sodium citrate. Further investigation led to the conclusion that the increase in activity was due to the removal of some inhibitor, probably cupric ion, from the solutions. A similar effect was found to be given by malonic acid and by oxalic acid.

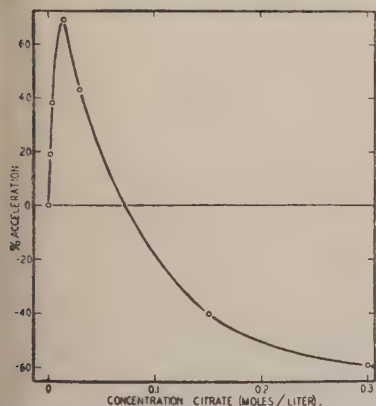


Fig. 1. The effect of increasing citrate concentration on the succinic dehydrogenase-cytochrome C system. $T = 38^\circ$, pH 7.15, succinate 0.06 m moles total, cytochrome C 11.5×10^{-6} m moles total, 0.1 cc of enzyme.

The rate of oxygen uptake of a system consisting of succinic acid, succinic dehydrogenase, cytochrome, and cytochrome oxidase was taken as a measure of the succinic dehydrogenase activity. The experiments were carried out in WARBURG-ERLENMEYER type flasks. The enzyme was prepared by a modification of the method of STOTZ and HASTINGS³⁾ in which the alternate washings with warm water were omitted. Such an enzyme preparation contained sufficient cytochrome oxidase to make the reduction of the cytochrome the limiting velocity in the system.

By comparing the total oxygen uptake to the total amount of succinate present it was determined that the acceleration of the rate of oxygen uptake resulting from the addition of citrate was not due to the coupled oxidation of the citrate by intermediate products formed in the oxidation of succinate. All the oxygen used could be accounted for by the succinate present, indicating that no citrate was oxidized. It also established that no enzyme having the ability to catalyze the oxidation of citrate was present.

Figure 1 illustrates the effect of various concentrations of **citrate** on the rate of oxygen uptake of the succinic-dehydrogenase-cytochrome system; low concentrations produce a marked acceleration; higher concentrations have an inhibitory effect. **Oxalate** and **malonate** give very similar curves, except that the maximum acceleration by malonate is reached when the concentration is only 3×10^{-4} molar, and complete inhibition is given by a malonate concentration of less than 1×10^{-2} molar. Thus malonate appears to be about 50

times as effective as citrate. Tartrate was found to have no accelerating influence, although it inhibited somewhat in higher concentration. Malic acid was found to be an efficient inhibitor but produced no acceleration.

With different enzyme preparations the effect of citrate, malonate or oxalate was of different magnitude. This was taken as an indication that traces of copper were introduced during the preparation of the enzyme. In several instances enzyme preparations were obtained which were not appreciably affected by low concentrations of the reactivating substances. It was possible to partially inactivate such preparations by adding traces of copper and subsequently restore the activity by adding citric or malonic acids. Higher concentrations of copper produced complete inhibition; in such cases even high concentrations of citrate gave little or no reactivation.

HOPKINS, MORGAN and LUTWAK-MANN²⁾ report that 40 γ of copper have only a very small inhibitory effect on 0.5 gms tissue, 100 γ being necessary to produce complete inhibition. In the studies described above, an amount of enzyme preparation corresponding to 0.71 gms of tissue was completely inhibited by 3×10^{-8} moles of copper (about 2 γ). This would indicate that the enzyme preparation has been considerably freed of copper-binding impurities present in the whole muscle.

BANGA and PORGES⁴⁾ have investigated the correlation between the power of various organic and inorganic substances to bind copper and their inhibitory effect on succinic-dehydrogenase, with a view to determining whether or not the enzyme might be a copper-proteid. They found a definite parallelism between the two properties, except that glycollic, tartaric and citric acids, which formed copper complexes, inhibited very little. Since in these experiments the activity was measured by means of the reduction time of methylene blue they cannot very well be compared to the present work. It was found here, however, that citrate and tartrate do exert a definite inhibitory effect if added in somewhat higher concentration than reported by BANGA and PORGES.

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